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ORIGINAL PAPER

Effect of body size on toxicity of zinc in neonates of four differently sized *Daphnia* species

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Abstract The sensitivity of neonates of four *Daphnia* species to zinc was tested in relation to their mean body size. These mean sizes of these four *Daphnia* spp were: *D. magna*, 0.813 ± 0.055 mm, *D. pulicaria*, 0.745 ± 0.063 mm, *D. pulex*, 0.645 ± 0.044 mm and *D. galeata*, 0.611 ± 0.058 mm. A positive relationship between EC_{50} (24, 48) values and neonates size was found. The smaller the size of the daphnid the higher was the sensitivity to heavy metal toxicity. For all tested species did the EC_{50} values decrease with time; the decrease was most marked for *D. magna* and the least for *D. galeata*. The EC_{50} values of *D. magna* were higher than would be expected on basis of its body size.

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Introduction

Fresh waters receive most toxic substances generated by industry as waste and released into the environment. Although aquatic ecosystems are equipped with a variety of physico-chemical and biological mechanisms to eliminate or reduce adverse effects of toxic substances, toxicants may evoke changes in development, growth, reproduction and behaviour, and may even cause death of freshwater organisms (Rand et al. 2003).

Water fleas are among the most preferred animals for laboratory toxicity testing. Within the genus *Daphnia*, large-bodied *D. magna* is the most frequently used; furthermore, two other relatively larger *Daphnia* spp., *D. pulicaria* and *D. pulex*, are occasionally used as well (Cooney 2003). Other *Daphnia* species are rarely ever employed in toxicity testing (Benzie 2005). *D. magna* is usually employed mainly because of its relatively larger size and larger number of offspring than in other species (Canton and Adema 1978). Although animals for laboratory tests are usually kept under standardized laboratory conditions to exclude possible environmental effects, the natural habitats and life history

strategies of *Daphnia* species may differ (Koivisto 1995).

There are many published studies that compare toxicity of selected toxicants to different species (e.g. Bianchini et al. 2002; Grosell et al. 2002), but only a few studies have investigated differences in sensitivity to toxicants in different cladoceran species in relation to body-size (Ferrão-Filho et al. 2000; Koivisto et al. 1992; Bossuyt and Janssen 2005). As far as we know, there are no published studies relating body size and zinc tolerance among different *Daphnia* species.

Zinc is ubiquitous in the aquatic environments, but because its manifold uses in industry, e.g. in manufacture of alloys, for galvanizing iron, in dry-cell batteries, it is also among the most common contaminants of surface waters due to human activity (Terrés-Martos et al. 2002; Van Sprang et al. 2004). It is an essential element, required as a cofactor for many enzymes. In general, the elevated exposure to essential as well as non-essential metal results in a non-specific binding of a reactive metal cations to biologically important macromolecules such as membrane receptors and enzymes causing modifications of their molecular function. The regulation of the distribution of trace metals among the various pools of macromolecules is central to maintaining metal homeostasis and optimal cellular function. At the cellular level, metabolism involves the binding of metals to inducible metal-binding ligands such as metallothionein and phytochelatin, which is an important way of eliminating zinc toxicity (Suzuki 1987; Goyer and Clarkson 2001; Di Giulio et al. 2003).

We tested the hypothesis that toxicity of zinc in different *Daphnia* species will decrease with increasing species size. To verify that, we determined the sensitivity of neonates of four differently sized *Daphnia* species to a range of zinc concentrations.

Materials and methods

Chemicals and reagents

ISO medium was prepared according to EN ISO 6341 (1998). All analytical grade chemicals ($CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$, $ZnSO_4 \cdot 7H_2O$, KCl) used for preparation of medium and stock solution of $ZnSO_4 \cdot 7H_2O$ were purchased from Lachner–Lachema (Czech Republic). Demineralized (conductivity less than 5 μS cm^{-1}) water was used to prepare stock solution of $ZnSO_4 \cdot 7H_2O$ immediately before start of tests.

Stock culture

Four differently sized species chosen in order of increasing mean body size were: *D. galeata*, *D. pulex*, *D. pulicaria* and *D. magna*. Each species was represented by one clone: *D. galeata* by clone TG100 (Repka et al. 1999), *D. pulex* by clone PXCL4 , *D. pulicaria* by clone PU3 and *D. magna* by clone HK (clone *a* sensu Baird et al. 1989; Vesela et al. 2006a). To prevent differences caused by acclimation to zinc, the animals were kept at a temperature of 20 ± 1°C and cultured in the same medium (40-μm sieve filtered pond water) for at least 3 months before start of the experiments.

To minimize maternal effects (Baird et al. 1989) only neonates originating from third to sixth brood females were used as mothers to produce test neonates. Mothers were kept in groups of 10 animals per 1,000 ml of medium at 20 ± 1°C, with a light:dark regime of 16:8 h and food level of c. 2 mg C l^{-1} of *Scenedesmus acutus* MEYEN.

Experiments

Neonates from third to sixth brood not older than 24 h were used in all tests. Test concentrations of zinc were subsequently prepared by adding an appropriate aliquot volume of stock solution $ZnSO_4 \cdot 7H_2O$ to ISO medium. Nominal concentrations ranged from 910 to 7822 μg $ZnSO_4 \cdot 7H_2O$ l^{-1} for tests with *D. magna*, 1050–3204 μg $ZnSO_4 \cdot 7H_2O$ l^{-1} for tests with *D. pulicaria*, 538–2563 μg $ZnSO_4 \cdot 7H_2O$ l^{-1} for tests with *D. pulex*, and from 430 to 2050 μg $ZnSO_4 \cdot 7H_2O$ l^{-1} for tests with *D. galeata*. It was ensured that there were enough treatments to get a good description of the whole dose-response curve. Per treatment and control, three replicates of 7 animals were used. Tests were carried out in 150 ml glass beakers containing 80 ml of the test solution, pH

of the test solution at the beginning of the tests was 7.8 ± 0.2 and the concentration of the dissolved oxygen was >7 mg l^{-1}. Experiments were run at light:dark regime of 16:8 h and 20 ±1°C for 24 and 48 h; no food was added during these periods. For more details of methodology and experimental conditions see EN ISO 6341 (EN ISO 1998) and Vesela et al. (2006b, c).

The aim of the tests is to determine the median effective concentration, EC_{50}, which is a concentration at which 50% of the exposed organisms are affected by measured effect (Newman 1995). In our tests, incapacitation of daphnids due to toxic action was chosen as main criterion to mark an adverse effect. Such animals are so immobilized that they cannot start swimming within 15 s after gentle shaking of the medium in the test beaker (EN ISO 1998).

Body mass approximation

Prior to toxicity bioassay 30 animals, not older than 24 h, were randomly selected from stock cultures of each clone. Their body length (*L*) was measured from top of the head to base of the tail spine under the microscope using a micrometer eye piece.

Data analysis

EC_{50} (24, 48) values and dose-response curves were calculated by non-linear regression using four parameter logistic equation (Motulsky and Christopoulos 2003) by the computer program GraphPad PRISM, version 4.0.

Results

Neonates of *D. magna* had the largest body-size (mean length 0.813 ± 0.055 mm), and those of *D. galeata* the smallest (mean 0.611 ± 0.058 mm), whereas those of *D. pulicaria* (mean 0.745 ± 0.063 mm) and *D. pulex* (mean 0.645 ± 0.044 mm) had intermediate sizes.

The relationships between per cent of juveniles immobilized and concentration of zinc for all *Daphnia* species and for both 24 and 48 h exposures are shown in Fig. 1. For all tested species at both exposure times dose-response curves obtained were sigmoid in shape. The curves observed for *D. galeata*, *D. pulex* and *D. pulicaria* were steep and of similar shape, whereas *D. magna* showed a more gentle increase with increasing zinc concentrations. The EC_{50} values obtained in tests for both exposure times increased with the increasing size of neonates (Fig. 2). The highest EC_{50} (24) value (mean, 95 % Confidence Limits, slope), was obtained for *D. magna* (mean size, 4,461 μg l^{-1}; range, 4,025–4,944 μg l^{-1}; slope 1.92), the lowest EC_{50} for *D. galeata* (mean size, 999 μg l^{-1}; range, 904–1,104 μg l^{-1}; slope 3.43), and *D. pulicaria* (mean size, 2,390 μg l^{-1}; range, 2,255–2,534 μg l^{-1}; slope 4.82) and *D. pulex* (mean size, 1,290 μg l^{-1}; range, 1,201–1,390 μg l^{-1}; slope 4.05) showed intermediate values. The toxicity effects increased with increase in exposure time from 24 to 48 h, with the most marked increase observed for *D. magna* and the least for *D. galeata* (Fig. 1). The highest EC_{50} (48) value was found for *D. magna* (2,254 μg l^{-1}, 2,254–2,587 μg l^{-1}, slope 1.86), the lowest for *D. galeata* (730 μg l^{-1}, 685–779 μg l^{-1}; slope 4.86),

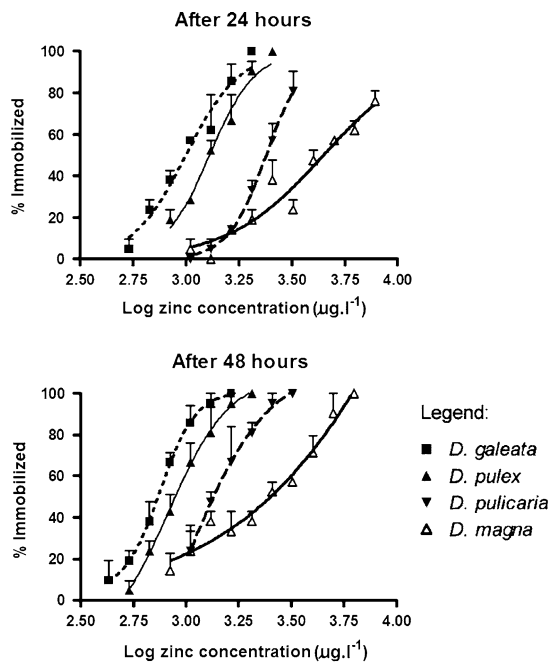

Fig. 1 Relationships between percentage inhibition in juveniles of *D. magna*, *D. pulicaria*, *D. pulex*, *D. galeata* and log zinc concentration for exposures lasting 24 and 48 h. Error bars represent ±1 SD

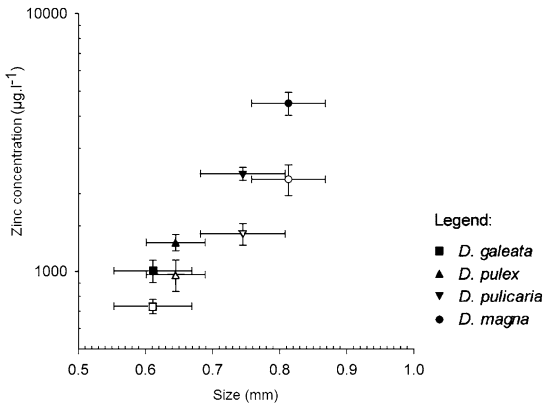

Fig. 2 Relationships between size of neonates and EC_{50} values in tests running 24 h (closed symbols) and 48 h (open symbols). Note log scale for EC_{50} concentrations. Error bars represent ±1 SD

and intermediate values were observed for *D. pulicaria* (1,373 µg l^{-1}, 1,245–1,514 µg l^{-1}; slope 4.12) and *D. pulex* (901 µg l^{-1}, 835–971 µg l^{-1}; slope 4.47).

Discussion

Toxicity bioassays are often used in aquatic ecotoxicology. The main objective of such tests is to determine the critical amount of toxicants or their mixtures for aquatic organisms and to predict a toxicant's influence and fate. Several standard methods for acute toxicity tests lasting 24 or 48 h, or both, and using water fleas as model organisms exist (Adams and Rowland 2003; Cooney 2003; Parrish 2003). In the present study, we used the standard EN ISO 3641 (EN ISO 1998).

Our observed EC_{50} values for *D. magna* compare well with those from literature (e.g. Van Sprang and Janssen 2001; Heijerick et al. 2002; Muyssen et al. 2002). For such comparisons, clonal variability and possible differences in test medium quality needs to be accounted for. The latter is important since hardness, alkalinity and pH of medium can all influence the speciation of metals and therefore the extent of toxicity (Barata et al. 1998; Heijerick et al. 2003). Unfortunately, we did not measure the zinc concentrations in the test medium. However, because a standard test medium was used consisting of four compounds only, and which had had a standard constant pH (EN ISO 1998), we do not expect any important changes in effective zinc concentrations due to a possible interaction with the medium.

According to EN ISO 3641 (1998) procedures toxicity bioassays are carried out without food so as to exclude the uptake of toxic substance adsorbed onto algae surface (De Schamphelaere et al. 2004). In the absence of food, the main route by which animals take up the toxicant is by contact with their body surface. As observed for cadmium, maximum carapace-adsorption potential is directly related to animal's surface area, but adsorption per unit of body surface is found to be similar under the similar exposure conditions (Robinson et al. 2003).

Zinc adsorbs onto animal's body surface and penetrates most probably by passive and facilitated diffusion (Rainbow 1995). It is generally accepted that the main uptake of zinc is related with calcium channels located in cell membrane (Wright 1995; Vercauteren and Blust 1999). The relationship between metal influx and accumulation rates in animal's body and its concentration in the dissolved phase of medium was described by Vercauteren and Blust (1999) and Yu and Wang (2002). For exposure to a given metal concentration, the influx rate of zinc increases with decreasing body size of animals. Once the zinc ions enter the body tissue, they interact with high and low-molecular weight ligands and metallothioneins as well as with other cellular compartments (Di Giulio et al. 2003). Metal ions bind also to special elements, which consequently start metallothionein synthesis (Kille et al. 1992).

The metabolic rate of smaller organisms is higher than those of larger ones. Since the metabolic rate has a positive effect on the accumulation rate of zinc (Newman and Mitz 1988; Yu and Wang 2002), the amount of accumulated metal ions per unit of body volume is higher in the smaller-sized species and, therefore, the latter species are more sensitive to acute toxicity than the larger ones (Grosell et al. 2002; Bianchini et al. 2002). Size-specific sensitivity to acute heavy metal toxicity, with the smallest individuals showing the highest sensitivity, was recently also observed within the same daphnid species (Bianchini et al. 2002). Thus, in our experiment,

except for the relatively low sensitivity of *D. magna*, the higher sensitivity of smaller individuals is probably mainly caused by size differences rather than by interspecific differences.

EC_{50} (24) values of *D. magna*, the largest species used in the present study, were approximately one-third higher than would be expected on basis of the relationship we observed between toxicity and the body size of the neonates of the other three species. The shape of the dose-response curves of *D. magna* in both 24 h in 48 h tests also differed from those of the smaller species. This could be an evolutionary adaptation: *D. magna* is ecologically adapted to small ponds and rock pools in which temporal changes in water chemistry are more rapid and the contaminant concentration are usually higher (Koivisto 1995). Such an environmental stress may facilitate tolerance to increased concentrations of contaminants. Important protective mechanism acting against heavy metal toxicity is detoxification of these metals through their chelation caused by binding to metallothioneins and other protein ligands (Guan and Wang 2004). *D. magna* species may have a larger amount of these proteins per unit of body weight, or their production after induction by metals could be higher than in other *Daphnia* species. This view is supported by our observation, which reveal that the toxic effect of zinc on *D. magna* individuals increased more slowly with increasing zinc concentrations in the medium than for other *Daphnia* species.

Only a few studies have investigated differences in sensitivity to toxicants in relation to body-size in different cladoceran species. Ferrão-Filho et al. (2000) examined the toxicity of microcystin present in *Microcystis* on the life history of six cladoceran species of which three were *Daphnia* spp. They found that within the cladocerans as a group there is no relationship between size and sensitivity for this toxin. However, among the three daphnid species, the largest (*D. similes*) and the second largest species (*D. pulicaria*) of the three were less sensitive, than *D. pulex* that is similar in size to *D. pulicaria*. These results do not agree with ours, but this may be because microcystin is an endotoxin, which in contrast to zinc has to be digested before it can affect the organism. Similarly to our study, Koivisto et al. (1992) investigated the sensitivity to copper of five cladoceran species of different body size, including three *Daphnia* spp. They found a clear relationship between size and copper tolerance, with the largest species showing the highest tolerance and the smallest species the lowest. Bossuyt and Janssen (2005) who investigated the sensitivity to copper in a several cladoceran species observed that large chydorids were more sensitive than small ones. In other families (Daphniidae, Bosminidae and Macrothricidae), however, it was just the other way round, i.e. smaller animals were more sensitive than larger animals. Both these the last-named studies support our findings on the relationship between body size and zinc toxicity because copper like zinc is an essential metal and behaves equally as zinc in many respects (Goyer and Clarkson 2001).

We tested only one clone per each of the 4 *Daphnia* spp, but it is known that daphnids may show clonal (i.e. genetic) variation in tolerance to essential metals toxicity (Baird et al. 1990; Barata et al. 1998). Barata et al. (1998) showed that EC_{50} (48) toxicity of zinc for clones of *D. magna* differed by factor 2.5-fold. In our present study EC_{50} (24 and 48) values among *Daphnia* spp. varied 4.6-fold and 3-fold, respectively. It is, however, difficult to say to what extent clonal variation would have affected our results had we used 4 clones per species instead of one clone per species. However, because both Koivisto et al. (1992) and Bossuyt and Janssen (2005) studies on the sensitivity to copper of non-chydorid, cladoceran species of different size support our results, we expect that use of more clones per species would not have changed our main results.

Conclusion

We found a positive relationship between EC_{50} (24, 48) and neonates body size, the smaller the size of the daphnid species the higher the sensitivity to heavy metal toxicity and vice versa. This is probably in the first place a size dependent effect irrespective of the species concerned. However, besides this size effect we also found a species specific effect. Size specific sensitivity to

heavy metal toxicity in *D. magna* was lower than would be expected on basis of its body size alone.

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